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Characterization of Yersinia Pseudotuberculosis Abiotic Stress Responses

Amanda Joyce Henry

CHARACTERIZATION OF YERSINIA PSEUDOTUBERCULOSIS ABIOTIC STRESS RESPONSES

THESIS

AMANDA JOYCE HENRY

2013

CHARACTERIZATION OF *YERSINIA PSEUDOTUBERCULOSIS* ABIOTIC STRESS RESPONSES

THESIS

Presented in Partial Fulfillment of the Requirements for

the Master of Science Degree in the Graduate School

of Texas Southern University

By

Amanda Joyce Henry, B.S.

Texas Southern University

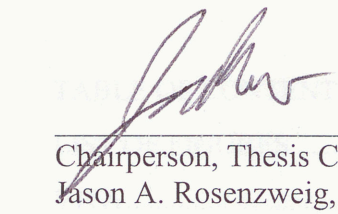
2013

Approved By:

Chairperson, Thesis Committee

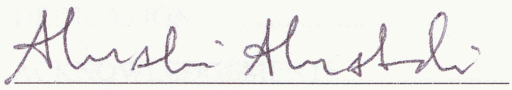
Dean, The Graduate School

Approved By



Chairperson, Thesis Committee
Jason A. Rosenzweig, Ph.D.

5/7/2013
Date



Committee Member
Shishir Shishodia, Ph.D.

5/7/2013
Date

Committee Member
Fawzia Abdel-Rahman, Ph.D.

5/7/2013
Date

Committee Member
Robert L. Ford, Ph.D.

Dean, the Graduate School
Gregory Maddox, Ph.D.

Date

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VITA

April 2, 1984.....Born, Port Arthur, TX

2002.....Monsignor Kelly Catholic High School

2006.....Sc.B., Texas Southern University
Houston, Texas

2007.....Certificate Business
Entrepreneurship, California State
University-San Bernardino, San
Bernardino, California

2009.....NASA CBER Fellow

2011-2012.....Teaching Assistant,
College of Science and Technology,
Texas Southern University

2011.....Graduate Student Tutor,
Student Academic Enhancement
Services, Texas Southern University

Publications

Henry, Amanda, Shanks, Justin, Rosenzweig Jason A., Van Hoof, Ambro,(2012) The Yersinia pseudotuberculosis degradosome is required for oxidative stress, while its PNPase subunit plays a degradosome-independent role in cold growth. FEMS Microbiol Lett 336 (2012) 139–147.

Henry, Amanda, Chopra Ashok K., and Rosenzweig, Jason A., "Space Flight", The Influence of Spaceflight and Modeled Microgravity on Bacterial Virulence, Kulshreshtha, Arun, Intech

Presentations

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Field of Study

Major Field: Biology (Microbiology)

DEDICATION

One must know from whence they came in order to embrace who they are as an individual. Keeping that theme in mind, this is dedicated to my grandparents, parents, my deceased personal mentor, my paternal grandparents (that made strides for blacks in our hometown), and my late maternal grandparents whom were both excellent educators. To my parents you trained me so that I would know the way to go. Words cannot contain the amount of gratefulness I have for you two. Also, in loving memory of Coach Robert Powell for always believing in me and doing everything he could to see to it that my academic progress would flourish. In closing, if it had not been for the Lord who was on my side this work would not have been completed. ~Amanda J. Henry

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CHAPTER 1

INTRODUCTION

The Gram-negative rod shaped bacterium *Yersiniae* belong to the Enterobacteriacea family. This genus consists of multiple species, in which only *Yersinia pestis*, *Yersinia entercolitica*, and *Yersina pseudotuberculosis* have the ability to cause human disease. A commonality between the pathogenic strains is the virulence plasmid (Darwin & Hortsman, 2012). When this plasmid is encoded it enables the Type Three Secretion System (TTSS) in combination with the proteins it secretes. This is one of many virulence factors that influence yersiniae infectivity however only becomes active in the presence of the host cell.

Yersinia pseudotuberculosis employs the TTSS to infect host cells, and polynucleotide phosphorylase (PNPase), a phosphorolytic 3′–5′ exoribonuclease involved in RNA decay, is required for its optimal functioning (Rosenzweig et al., 2005, 2007). PNPase is required for the cold-shock response and/or acclimation for a number of organisms including

Yersinia pestis and *Y. pseudotuberculosis* (Rosenzweig et al., 2005, 2007), *Escherichia coli* (Jones et al., 1987; Mathy et al., 2001; Yamanak & Inouye, 2001; Polissi et al., 2003), and *Yersinia enterocolitica* (Goverde et al., 1998; Neuhauset al., 2000; Neuhaus et al., 2003). Intriguingly, PNPase has been shown to physically interact with an essential endoribonuclease, RNase E, in both Escherichia coli (Carpousis et al., 1994; Vanzo et al., 1998; Khemici & Carpousis, 2004) and *Y. pseudotuberculosis* (Yang et

al.,2008) forming a large multiprotein RNA surveillance/quality control complex termed the degradosome.

E. coli established Ribonuclease E, polynucleotide phosphorylase (PNPase), the deadbox RhlB helicase, and enolase (a glycolytic enzyme), as the large protein complex, the degradosome. (Jones et al., 1987) Typically, a degradosome consists of both an exo- and endoribonuclease (e.g. PNPase and RNase E), and they are thought to work together in concert producing a synergistic effect that optimizes RNA decay of unwanted transcripts. (Henry et al., 2012) However, a degradosome consisting of both RNase R, a cold-inducible exoribonuclease in E. coli (Cairrão et al., 2003; Chen & Deutscher, 2010) required for the maturation of SsrA/tmRNA (Cairrão et al., 2003), and RNase E has also been identified in the psychrotrophic Pseudomonas syringae, possibly suggesting the existence of a specialized cold-adapted degradosome (Purusharth et al., 2005).

It is uncertain as to whether Y. *pseudotuberculosis* PNPase plays a role in the oxidative stress response through a degradosome-dependent mechanism; PNPase's role during cold stress is degradosome-independent(Henry et al., 2012). This study examined the abiotic stress factors experiences by *Yersinia pseudotuberculosis*, since the majority of these studies have been conducted in *Escherchia coli* (*E. coli*). Polynucleotide phosophorlytic (PNPase) will be instrumental in delineating the mechanisms whereby cold/oxidative stress factors influence protein structure in both *Escherichia coli* and *Y. pseudotubercuoslis* (Henry et al., 2012; Jones et al., 1987; Palononen et. al, 2012; Wu, 2008).

CHAPTER 2

HISTORY AND LITERATURE REVIEW

2.1 Pathogenic *Yersinia*

The yersiniae are Gram-negative, facultative anaerobes that belong to the Enterobacteriacae family (Darwin & Hortsman, 2012). The optimal temperature in which the *Yersiniae* grow is 4°C to 40 °C degrees (Zartarsh et al. 2012). There are 11 species within the genus, and of those 11 there are three3 human pathogens (Zartarsh et al. 2012). Examples of both non-pathogenic and pathogenic species that are related to *Yersinia* include *Escherichia*, *Vibrio*, and *Shigella*. (Bent et al. 2010, Erce et al. 2009, Lawal et al. 2010) *Yersinia pestis* (YP) is a derivative of (YPT) (Achtman et al., 1999) and like *Yersinia entercolitica* (YE). Yersinia *pestis* is the causative agent of plague. Whereas, *Yersinia entercolitica* and *Yersinia pseudotuberculosis* are not as fatal due to their pathogenesis in comparison to *Yersina pestis* due to its pathogenicity. A flea is the actual arthropod that serves as the mechanical vector for YP to enter the human body. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* enter the body via the oral-fecal route and cause ailments such as sepsis, diarrhea, yersiniosis, and mesenteric lymphadentis to name a few (Galindo et al., 2011). This is caused by the vertical transmission from food or water sources or hand to mouth that typically derives from feces of farm animals. This plays a key role in the virulence potential of disease in a given host cell. It is important to note the role of the host cell in immune response specifically as it relates to first line of defense (Lawal et al., 2010a). In order, for a

Yersinial infection to enter into its host, a $10^{\wedge 9}$ inoculum is necessary (Zartash et al., 2012). The host cell after invasion of *Yersinia pseudotuberculosis* and *Yersinia entercolitica* has to fight infection against dendritic, neutrophils and macrophages for example to prevent it from spreading (Schiano & Lathem, 2012). Additionally, research has shown that the intestinal tract also plays a key role in bacterial replication, and that the sole source is not simply the lymphatic system. In short, the aforementioned points collectively play a role in *Y. pseudotuberculosis* pathogenesis which affects the liver, spleen, and lymph nodes and can have adverse affects on immune-compromised people (Galindo et al., 2011; Zartarsh et al., 2012).

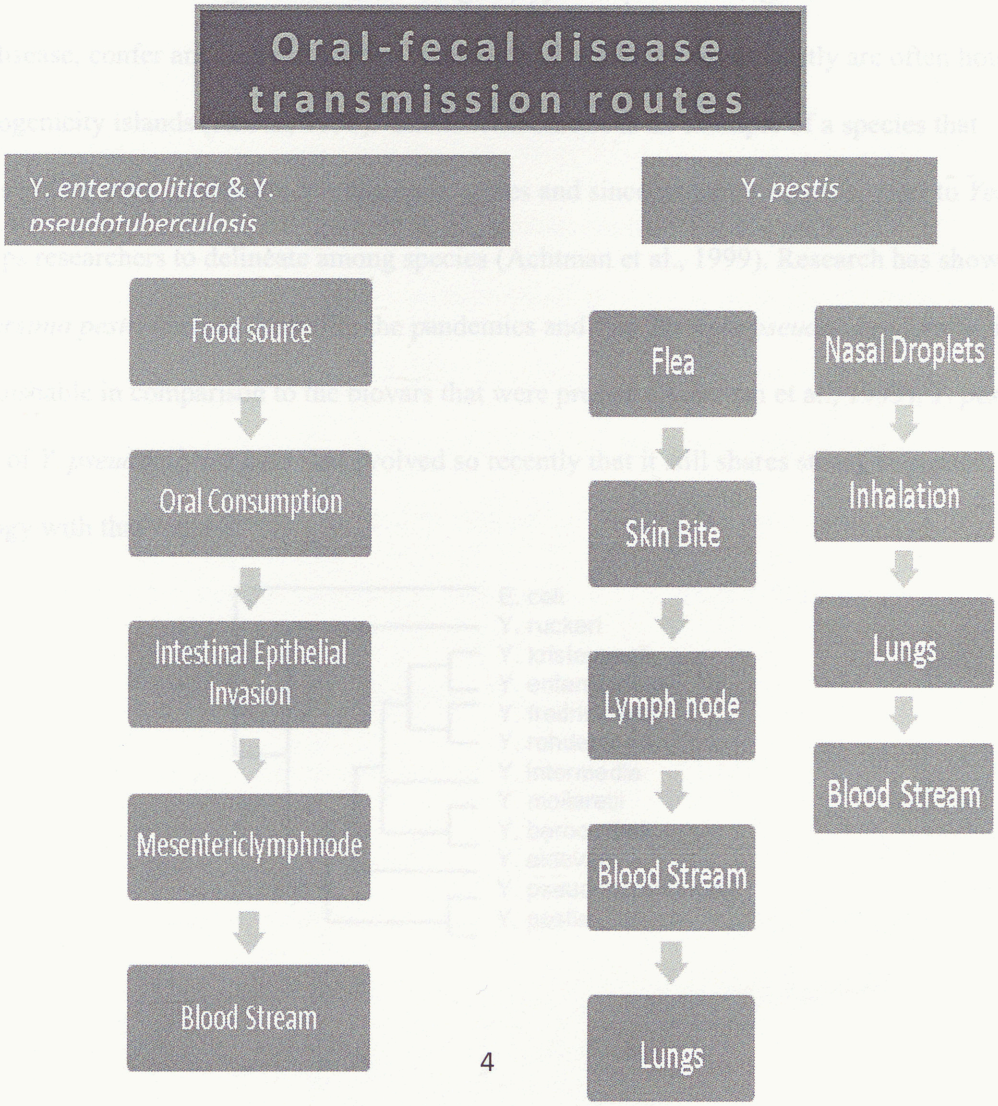

Figure 1 Oral-fecal route

Above is a schematic of how pathogenic strains enter into the body. *Yersinia entercolitica* and *Yersinia pseudotubercolsis* derive from sources such as drinking/waste water, soil, or contaminated food. Due to the natural of these species they rarely enter into the blood stream unless the individual is immunocompromised. Whereas pathogenic YP is able to enter its host cell via a flea vector and cause a pandemic (modified Darwin and Hortsman, 2012).

2.2 Phylogenecity of *Yersinia*

In order to correlate the significance of this study phylogenics is necessary in order to connect the dots of species evolution in particular *Yersinia*. As previously mentioned only three of the 11 strains of *Yersinia* are pathogenic. What distinguishes pathogenic from non-pathogenic species is the concept of horizontal transfer. The principle of horizontal transfer of genes into an organism that is not its original donor and the gene types acquired through this mechanism can cause disease, confer antibiotic resistance, capable to degrade toxins and lastly are often housed in pathogenicity islands (Hueck, 1998). *Salmonella* enteric is an example of a species that displays phylogentic history via housekeeping genes and since its homology is similar to *Yersinia* this helps researchers to delineate among species (Achtman et al., 1999). Research has shown that, *Yersinia pestis* derived thereafter the pandemics and that *Yersinia pseudotuberculosis*. is distinguishable in comparison to the biovars that were present (Achtman et al., 1999). *Y. pestis* is a clone of *Y. pseudotuberculosis* that evolved so recently that it still shares strong sequence homology with that species.

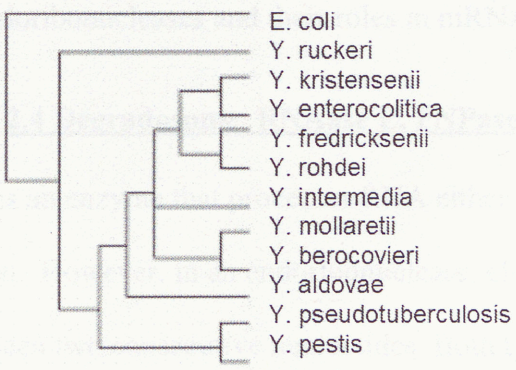

Figure 2 Yersinia Phylogenic Tree

Demonstrates the pathogenic evolution within the *Yersinia* genus in a phylogenic manner and how horizontal gene transfer caused said change (Bent et al., 2010; Chen et al., 2010).

2.3 RNA Decay

The central dogma involves trascribing information from DNA to RNA for ultimate translation into a functional protein. Decades ago, it was thought that ribonucleases, enzymes that degrade RNA into smaller fragments, was not specific and its role in said processing was not definitive (Lawal et al., 2010a; Deutscher et al., 2001;Kushner et al., 2002). However, more recent studies state that different species of RNA are involved on temporary enzymatic controlled events. *E.coli* is the model organism in which RNases have been studied; these RNAases have been attributed to having virulence factor characteristics in both gram- negative and gram-positive organisms. For instance, studies have been conducted in gram negative bacteria such as *Pseudoalteromonas haloplanktis* (Ait-Bara & Carpousis, 2010) and in gram positive bacteria such as Bacillus *subtilis* (Burger et al., 2011). However, there is some background information that is needed to make that connection, specifically exoribonucleases and endoribonucleases and their roles in mRNA degradation.

2.4 Degradosome, RNAase E, PNPase

An exoribonuclease is an enzyme that processes RNA either in the 3' to 5' direction or in the 5' to 3' direction. However, in an endoribonuclease, cleavage takes place in the middle of the RNA between two consecutive nucleotides. Both types of ribonucleases are found in *E.coli*. The degradosome is a multi-protein complex composed of several

proteins including: Ribonuclease E (RNase E), Polynucleotide phosphorylase (PNPase) RhlB (RNA helicase), and enolase (Henry et. al, 2012; Ait-Bara & Carpousis, 2010; Carpousis, 2002; Khemici & Carpousis, 2004; Lawal et al., 2010). Required for viability of *E. coli* , RNAase E containing the temperature sensitive mutant ams-1 (altered mRNA stability), which manages the rate of decay during the first endonucleolytic cleaveage (Munkegekar, 2009; Rosenzweig et al., 2005).

The RNAase E protein is 1061 amino acids in length and 118 kDa is mass. The structure of the degradosome is as follows: The first set of amino acids 1- 498 is at the N terminus or amino terminus and encompasses the S1 RNA binding domains, the second half, the carboxy terminus half (CTH) unlike its counterpart is not necessary for growth. In the CTH, spanning residues 734-1060 act as a scaffolding region which contains the previously mentioned microdomains , PNPase, RNAase PH(a resemblance of one of PNPases' catalytic cores) the RNA helicase B (RhlB or the DEAD box), and the gylcoytic enzyme enolase (Rosenzweig et al., 2005; Lawal et al., 2010a). Additionally, findings have shown that the C-terminus of the complex is divergent amongst other genras (Burger et al., 2011; Lawal et al., 2010a; Carpious, 2002; Munegekar, 2009). There are several additional enzymes and minor proteins found within the degradosome such as RNA G, III, P, and DnaK, GroEL proteins (Lawal et al., 2010a; Munegekar, 2009; Carpious, 2002). As this study is focused on the degradosome the aforementioned minor proteins will no longer be discussed.

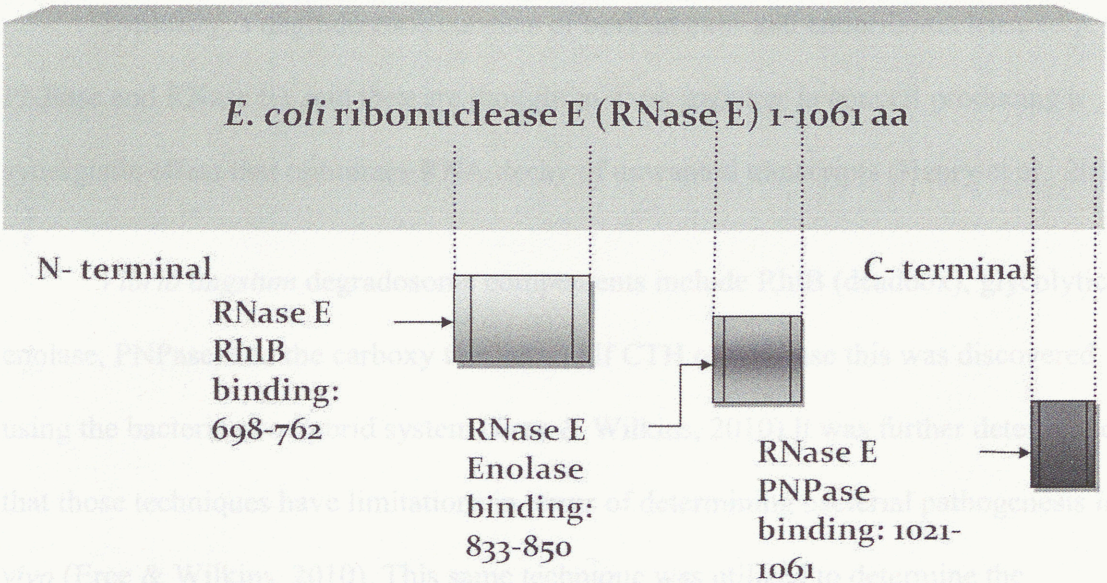

Figure 3 Degradosome

A schematic of the *E. coli* degradosome. The N-terminus contains the first 1-498 residues. The carboxy terminus half (CTH) is (499-1061) residues of the RNAase E protein. The binding sites housed in the CTH are as follows: RhlB (698-762), Enolase (833-850), and PNPase (1021-1061). (Burger et al., 2011; Lawal et al., 2011).

The primary function of the degradosome is to provide stability, process mRNAs, and it also serves as a backbone to the entire multifunctional protein complex (Burger et al.,2011). Furthermore, proteins of the catalytic enzyme degrade mRNA and process rRNA that act upon the scaffolding protein with all the aforementioned subcomponents that improve cell processing and degradation (Lawal et al., 2010a). Subunits enolase, RhlB dead-box, and PNPase assemble at the scaffolding region at residues 833-850, 698-762, and 1021-1061(Burger et al., 2011)

2.5 Degradosome Variation Among Species

Typically, a degradosome consists of both an exo- and endoribonuclease (e.g. PNPase and RNase E), and they are thought to work together in concert producing a synergistic effect that optimizes RNA decay of unwanted transcripts (Henry et al., 2012).

Vibrio angstum degradosome compentents include RhlB (deadbox), glycolytic enolase, PNPase, and the carboxy terminus half CTH of RNAase this was discovered using the bacteria two hybrid system (Erce & Wilkins, 2010).It was further determined that those techniques have limitations in terms of determining bacterial pathogenesis *in vivo* (Erce & Wilkins, 2010). This same technique was utilized to determine the components in the YPT degradosome (Henry et al., 2012). Another psychrophilic organism, *Pseudoalteromonas haloplanktis,* is derviced from the Antarctic ocean and is of interest to the cold marine industry; its degradosome is composed of the same *E. coli, V. angstum*, and *Y. pseudotuberculosis* counterparts with the exception of enolase (Ait-Bara & Carpousis, 2010; Erce & Wilkins, 2010; Henry et al., 2012, Rosenzweig et al.,2005).

2.6 Abiotic Stress Exposure to the Degradosome

At present it is known that cells regulate metabolism and environmental conditions and physical requirements can potentially alter the structure of a protein, in this case the degradosome. As previously mentioned the host cell immune response links together the environmental factors involved ex vivo and can have infectious effect once the TTSS is activated (detailed in future studies/recommendations) (Chowdhury et al., 1996). However, these growth conditions are relevant in either case. Other factors such as heat shock, osmolarity are capable of altering pathogenic spread at the translational

and transcriptional level (Chowdhury et al., 1996; Darwin & Hortsman, 2012; Schiano & Lathem, 2012). RNAs are said to be a better energy source in comparison to proteins for gene expression according to the literature (Gripeland et al., 2010). In particular to this project the stress factors that were evaluated included both cold growth and oxidative stress and their roles in the yersiniae degradosome.

In keeping with the beginning of this work *E. coli* as well as *B. subtilis* are the two main strains in which cold shock studies have been conducted (Goverde et al., 1998; Lawal et al., 2010a; Burger et al., 2011). Temperature is one of those factors that have an effect on enzymatic activity and shape. In the model organism *E. coli* its temperature range is 20° to 40° therefore categorizing it as a mesophile and it mimics patterns in the human body due to its pathogenesis. Whereas the yersiniae are psychrophiles and have different growth conditions (temperature) that effect proliferation and expression and will be discussed later. An unexpected drop in temperature of 10 ° that bacterium encounter is known as "cold shock response" (Henry et al., 2012; Jones et al., 1987; Lawal et al., 2010a; Mathy et al., 2001; Polissi et al., 2003; Rosenzweig et al., 2005). The basic mechanism involved in cold shock response is to allow a given strain to adapt to downshift due to its involvement in cellular processing (Palonen et al., 2010). Additionally, cold stress can alter the cell's environment by the following metrics: enzymatic activity, the influx and outflow of the membrane specifically its fluidity, and protein topology to name a few factors (Polissi et al., 2003). Furthermore, studies have shown where protein synthesis varies between mesophilic strains vs psychotrophic bacterium (Jones et al., 1987; Neuhaus et al., 2000; Palonen et al., 2010). Therefore, the

growth rate is slowed down significantly due to the temperature change and thus causing genetic reprogramming (Jones et al., 1987; Palonen et al., 2010). These changes are said to occur during the lag phase of a growth curve and E. *coli* PNPase is said to downregulate (Jones et al., 1987). However, a degradosome consisting of both RNase R, a cold-inducible exoribonuclease in *E. coli* (Cairrão et al., 2003; Chen and Deutscher, 2010; Henry et al., 2012) required for the maturation of SsrA/tmRNA (Cairrão et al. 2003; Henry et al., 2012), and RNase E has also been identified in the psychrotrophic *Pseudomonas syringae*, possibly suggesting the existence of a specialized cold adapted degradosome (Henry et al., 2012; Purusharth et al., 2005). There are a couple of sets of particular genes that are involved in cold shock. The first being cold shock proteins (Csp) which aid in cold adaptability and the other is cold inducible proteins (Cips) (Jones et al., 1987; Palonen et al., 2010; Yamanka & Inouye, 2001). The *pnp* gene is attributed with cold growth conferral in yersiniae, E. *coli*, and B. *subtilis* to name a few (Goverde et al., 1998; Lawal et al., 2010a; Rosenzweig et al., 2005). The role of CIPs are to serve as a nucleic acid-binding proteins that's functionally is attributable to cell regrowth upon acclimatization and helps stabilize both RNA and DNA secondary structures (Palonen et al., 2010; Polossi et al., 2003). Two of the primary csp genes are *cspA*1 and *cspA*2 and their function is a coding region that assists in adaptability in YE. (Palonen et al., 2010; Neuhaus et al., 2000).

2.7 Post-cold shock/ Acclimization

The physical state of the cell is altered on the ribosomal and nucleic acid levels in response to said environmental change. Upon termination of cold shock response, RNAase E cleaves the csp transcripts into smaller units while mRNA degradation is

carried out via PNPase. After that step the number of ribosomes present is so low that growth no longer is possible and is regulated via a cspA gene known as the cold-box (AGUAA) and is said to behave similarly in E. *coli* and *Yersinia* (Jiang & Belasco, 2000; Neuhaus et al. 2003, ; Palonen et al., 2010). As previously mentioned E. *coli* and YE get rid of useless csp transcripts in order to continue degradation and restore pre-stress expression (Lawal et al., 2010a; Palonen et al., 2010; Goverde et al., 1998; Nehaus; Yamanka & Inouye, 2001; Polissi et al., 2003; Rosenzweig et al., 2005). Cips are what allows acclimatization to occur (Yamanka & Inouye, 2001). In yersiniae, S1 binding domains are necessary for cold growth (Rosenzweig et al., 2005; Rosenzweig et al., 2007).

To date the literature has shown that PNPase is needed for post cold shock in *E. coli,* however for this particular work we wanted to determine if PNPase's role in cold growth was independent or dependent in YPT. In order to make this determination one must factor into consideration studies that were conducted in the host cell to determine PNPase's role specifically with the TTSS (Rosenzweig et al., 2005). Other gram-negative strains (*Salmonella* and B *subtilis*) indicated in the literature that in fact PNPase is required and research confirmed that PNPase is needed for YPT cold growth as well (Goverde et al., 1998; Neuhaus et al.,2000; Yamanka & Inouye, 2001; Rosenzweig et al., 2005). In short there are several similarities in yersinae in comparison to *E. coli* which include PNPase metabolizing and reprogramming under cold shock, the S1 domain influence on Gram-negative strains in cold growth, and lastly it aids sustainability in the host cell (Rosenzweig et al.,2005). Researched has been conducted to show that carboxy-

terminally truncated RNAase E conferred a dominant-negative expression and could stop due to its capability to autoregulate (Briegel et al., 2006; Henry et al., 2012; Yang et al., 2008). There is over a 90% homology between the first 500 residues in *E. coli* and YPT and the pBAD- rne 1-465 promoter was transformed into YPT and was shown to have an inhibitory effect on its functionality (Yang et al., 2008). The findings will able to establish that there was in fact a twofold relationship being functional and physical in nature in both RNAase E and PNPase (Yang et al., 2008). Therefore this laid the foundation for this work we will show the relevance of the degradosome and its subnits, in particular, PNPase and how this truncation played a role under stress response will be discussed in the results section.)

2.8 Oxidative Stress

Since PNPase has been proven to play a role in cold stress in E. *coli* and YPT it is safe to explore the possibilities it plays in an oxidative stress environment. The literature alludes to mentioning that any given stress environment will release its own particular set of proteins relative to that stress and those same proteins can further be induced by thermal shock or starvation for example (Farr & Kogoma, 1991) Oxidative stress is when a given living cell in this case a given microorganism is unable to detoxify or repair damage caused by a reactive oxygen species (ROS) which are oxygen containing molecules that are highly reactive. There are three forms of ROS which include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-).(Macvanin and Hughes). In the mitochondria of the eukaryotic oxidative phosphorylation happens whereas microorganisms lack intracellular membranes (Luschchak, 2001). When the threshold for homeostasis is exceeded from oxygen toxicity

this causes the cell to fight properly against the environmental change (Farr & Kogoma, 1991). Due to upregulation from ROS takes place as a defense or repair mechanism (Wu et al., 2008). Furthermore, the state of the cell is altered due to free radicals (O_2^-), or peroxide that is not processed via the electron transport chain as a final electron acceptor thereby causing toxicity. Consequently it causes damage on the DNA, protein oxidation to amino acids and oxidation of lipids (Farr & Kogoma, 1991; Hasset & Cohen, 1989). This in turn aids in disease that effects cells, tissues, organs, and organ systems causing ailments that are age related and cancerous in nature (Wu et al., 2008). Although many efforts (carcinogen and chemotherapeutic agents) are geared towards finding probable solutions to the aforementioned causes unfortunately most of those studies do not involve RNA oxidation (Bellacosa & Moss, 2003).

Aerobic respiratory organisms specifically microorganisms must protect themselves from ROS accumulation. There are less than five bulk enzymes that serve as protection although significant this is not the focus of this work (Farr & Kogoma, 1991). Over 100 genes were analyzed to delineate the over expression in a hydroxyl radical and superoxide manner (Macvanin). The 8-oxo-G lesion in *E. coli* is attributed with identified and detoxing RNA molecules influenced by PNPase (Wu et al., 2008). In *E. coli* PNPase is said not to protect the degradosome in the oxidative stress environment (Khemci & Carpousis, 2004; Wu et al., 2008). The truncated RNAase E mutant iis said to behave in a degradosome-indepeant manner in the presence of hydrogen peroxide (Lawal et al., 2010a; Wu). However, YPT is said to behave alternatively. (See discussion) Temperature and pH are two forms of envirmonemental conditions that are relative in terms of maintain stability within the yersiniae species (Schiano & Lathem, 2012).

Therefore, we sought to characterize the *Y. pseudotuberculosis* degradosome further because only PNPase has been shown to physically interact with RNase E (Yang et al., 2008) and to determine whether it is required for various abiotic stress responses (Henry et al., 2012).

Specific Aim

Evaluate the role(s) played by the yersiniae degradosome in cold and oxidative stress responses.

CHAPTER 3

DESIGN OF STUDY

3.1 Materials

YPT YPIII pIB102 (Bölin and Wolf-Watz 1984) (WT) and YPIII pIB100Δ*pnp* (Rosenzweig et al. 2005) were used for the cold growth and H_2O_2 plate-based assays.The arabinose-inducible promoter containing pBAD24 (Guzman et al., 1995) plasmid was used as a cloning vector into which a carboxy-truncated RNase E (encoding only the first 465 amino acid residues in the amino terminus) was cloned (Yang et al., 2008). For all inductions 0.02% arbinose was used unless otherwise noted. Ampicillin working concentrations were 100µg/mL.

3.2 Methodology

Cold growth assay

A previously published protocol (Rosenzweig et al., 2005) was used with several modifications. In short, 10-fold serial dilutions of saturated bacterial cultures were spotted in duplicate in ~ 2µl volumes (using a pronger) on 2 LB agar (Difco) plates containing 100µg/mL ampicillin (Sigma) and 0.02% arabinose (Sigma). One plate was placed at 30˚C while the other was placed at 4˚C and monitored for an 11 day period. Alternatively, cultures were streaked out on the aforementioned plates and monitored for their growth over an 11 day period (Henry et. al 2012).

H_2O_2 plate and liquid-based assays

Previously published protocols (Wu et al., 2009) were employed. In short, saturated cultures were diluted, and subcultures of OD 600_{nm} ~0.2 were established in triplicate 100µl volumes of LB medium (Difco) in 96 well plates. Following static growth @ 30˚C for 1.0 hours (with the appropriate antibiotic added and arabinose @ 0.02%), a stock 0.88 M H_2O_2 was added to the various cultures yielding H_2O_2 concentrations of either 0mM, 20mM, 50mM, or 100mM, respectively. Growth in the liquid cultures was monitored every 30 minutes over a 12 hour period with continuous agitation. Growth curves were plotted, and the Student's T-test was used to determine statistical significance with p values < 0.05 considered significant.

For plate-based H_2O_2 assays, 10-fold serial dilutions of saturated bacterial cultures were spotted in duplicate (using a pronger) in ~2µl volumes on 2 LB agar (Difco) plates containing 100µg/mL Ampicillin (Sigma) and 0.02% Arabinose (Sigma). Plate H_2O_2 concentrations were 0mM, 0.4mM, 1mM, 2mM, 4mM, and 100mM (Henry et al).

Pilot study

YPT pBAD24

YPT pBAD24 $_{+\Delta}$ *pnp*+ $RNE_{1\text{-}465}$

YPT pBAD24 $_{+}$ Δ *pnp*

YPT pBAD24 $RNE_{1\text{-}465}$

Figure 4 Cold Growth Pilot Study

All four samples were streaked out and grown for 11 days at 4 degrees. The YPT $pBAD_{24}$ +Δ*pnp* has less density in comparison to the other samples.

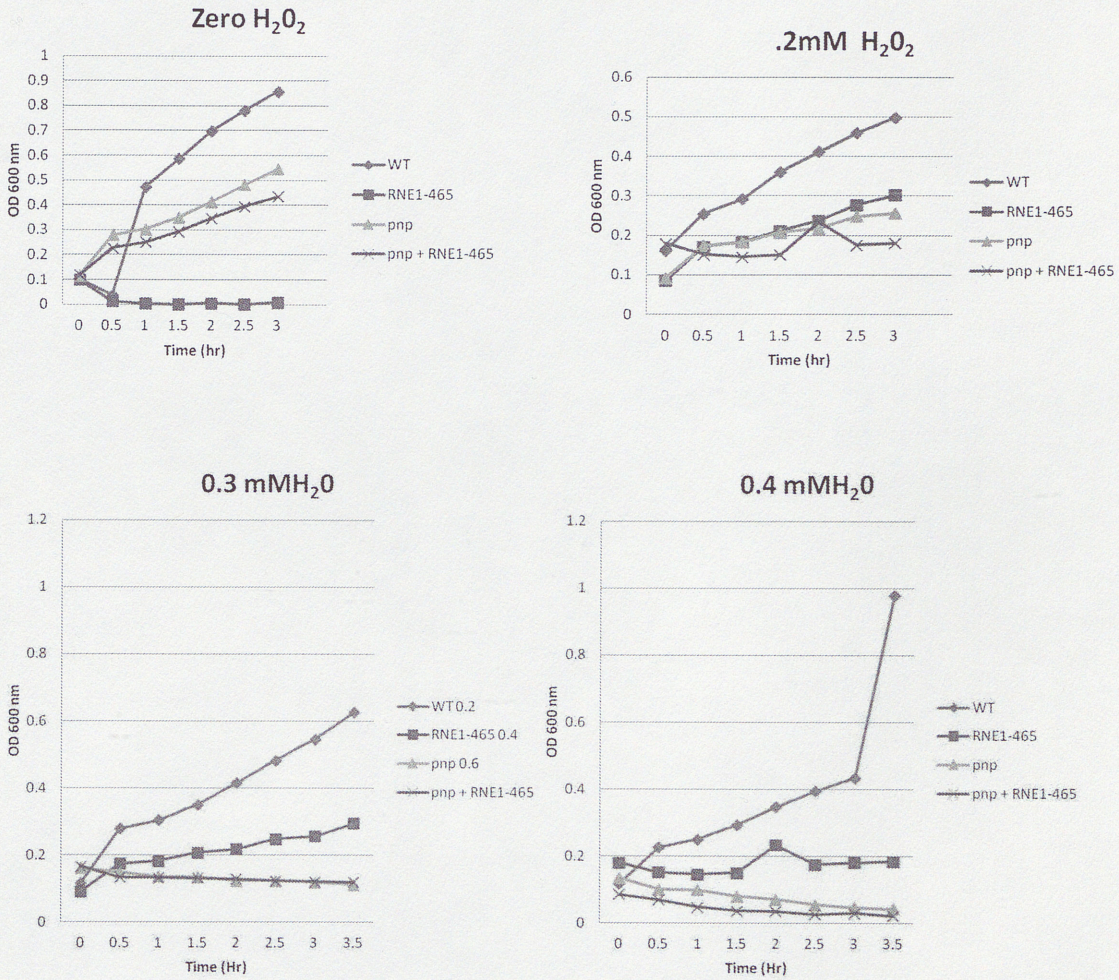

Figure 5 Growth Curve Pilot Study

The pilot study revealed that the wild type behaved the same regardless of the hydrogen peroxide concentration.

CHAPTER 4

RESULTS AND DISCUSSION

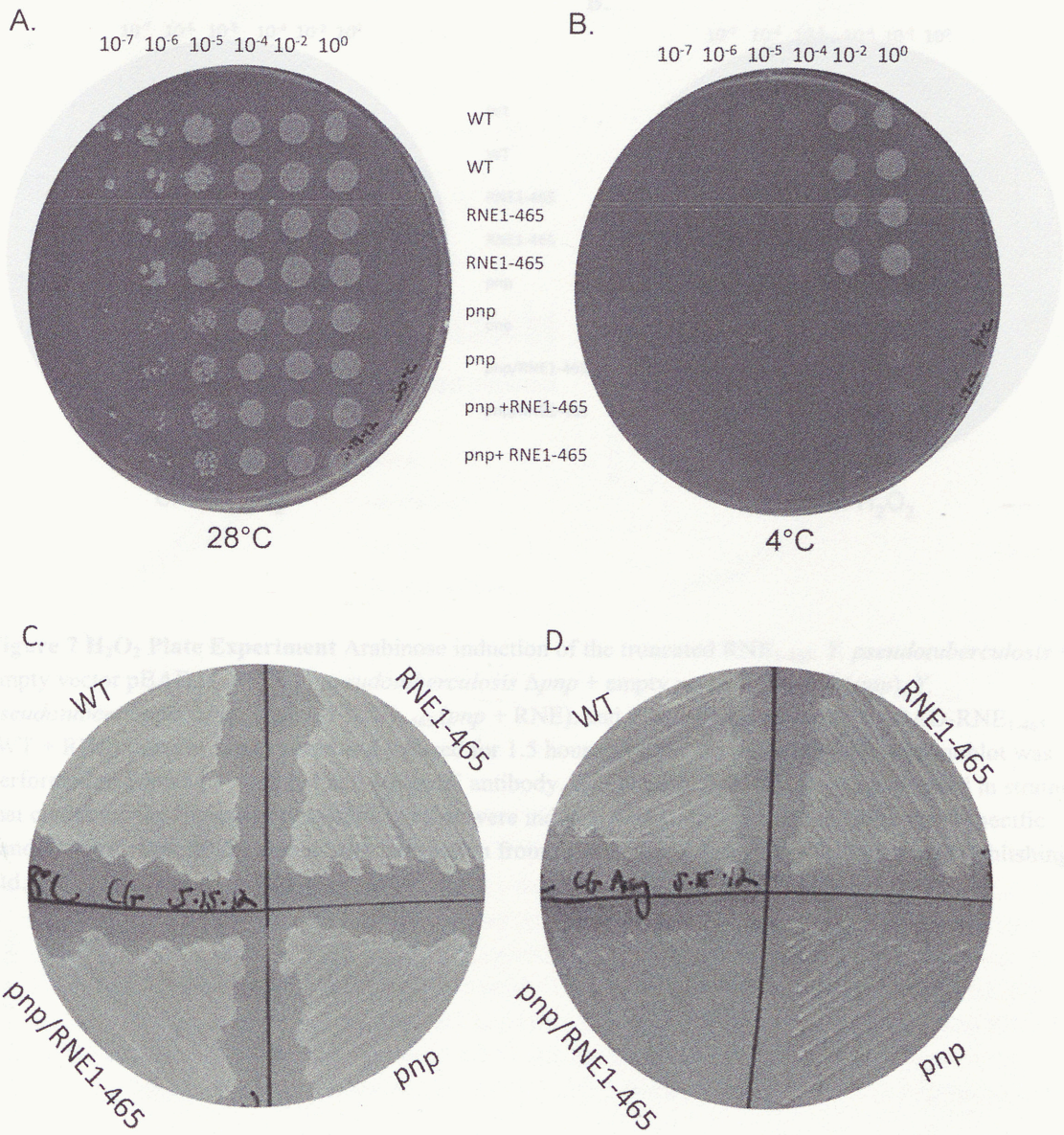

Figure 6 Cold Growth Experiments Representative cold-growth experiments. A. Various dilutions of saturated *Y. pseudotuberculosis* + empty vector pBAD24 (WT), *Y. pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (pnp), *Y. pseudotuberculosis* Δ*pnp* + pBAD-$RNE_{1\text{-}465}$ (pnp/RNE), and *Y.*

pseudotuberculosis + pBAD-RNE_{1-465} (RNE) cultures were spotted (using a pronger) on LB agar plates and grown at 30°C for 16 hours (A) or at 4°C for 11 days (B) at which point the plates were scanned. The same aforementioned strains were streaked on plates and images were acquired following 16 hours of growth at 30°C (C) or following 11 days of growth at 4°C (D). (Henry et al., 2012) copied with permission from FEMS Microbiology Letters Blackwell Publishing Ltd.

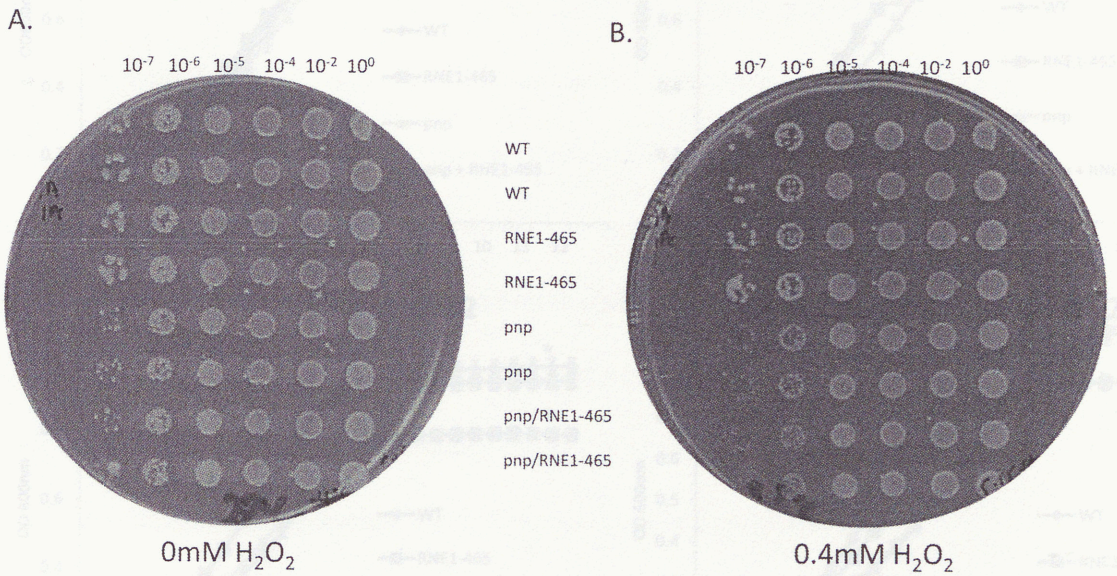

Figure 7 H_2O_2 Plate Experiment Arabinose induction of the truncated RNE_{1-465}. *Y. pseudotuberculosis* + empty vector pBAD24 (WT), *Y. pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (*pnp*), *Y. pseudotuberculosis* Δ*pnp* + pBAD-RNE_{1-465} (*pnp* + RNE), and *Y. pseudotuberculosis* + pBAD-RNE_{1-465} (WT + RNE) cultures were grown and induced for 1.5 hours with 0.02% arabinose. An immunoblot was performed employing polyclonal anti-RNase E antibody. CTD-deficient RNE_{1-465} was expressed in strains that contained the appropriate plasmid and that were induced with 0.02% arabinose. NSB= non-specific band. (Henry et al., 2012) copied with permission from FEMS Microbiology Letters Blackwell Publishing Ltd.

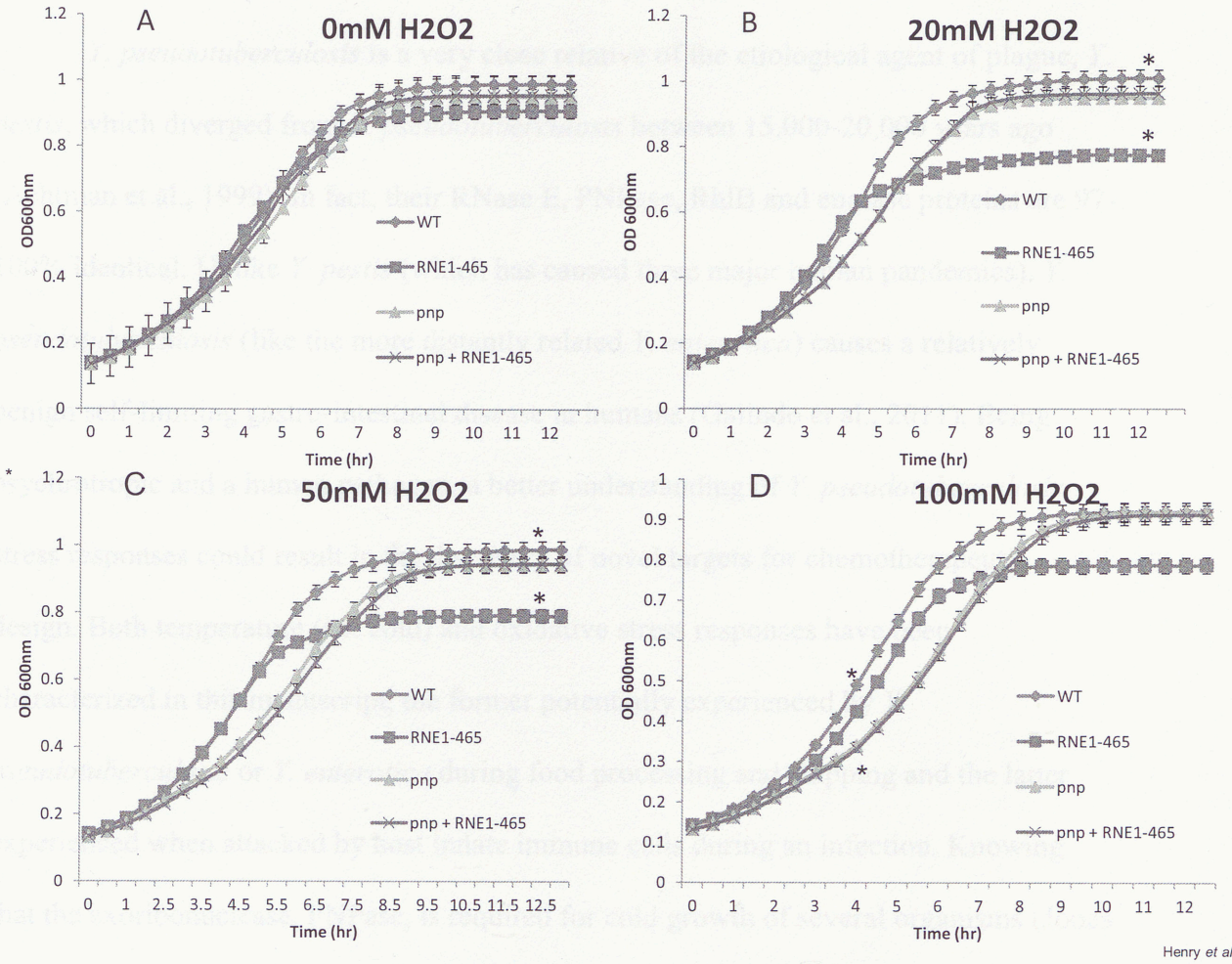

Figure 8 H2O2 Liquid Experiment Representative H_2O_2 liquid growth experiment. Subcultures of Y. pseudotuberculosis + empty vector pBAD24 (WT), Y. pseudotuberculosis Δpnp + empty vector pBAD24 (pnp), Y. pseudotuberculosis Δpnp + pBAD-RNE1-465 (pnp/RNE), and Y. pseudotuberculosis + pBAD-RNE1-465 (RNE) were grown (in triplicate) in 100 lL volumes using a 96-well plate. Following 1 h of static growth, 0 mM (a), 20 mM (b), 50 mM (c), or 100 mM(d) H2O2 was a added to the appropriate wells, and plates were constantly agitated while grown at 30 °C for 12 h. Samples were read at optical density 600nm every 30 min. Asterisk between the WT and pnp samples in d at 4 h and between WT and RNE in b and c at 12 h denote statistical significance ($P < 0.5$). All statistical tests employed the Student's t-test. (Henry et al., 2012) copied with permission from FEMS Microbiology Letters Blackwell Publishing Ltd.

Y. pseudotuberculosis is a very close relative of the etiological agent of plague, *Y. pestis*, which diverged from *Y. pseudotuberculosis* between 15,000-20,000 years ago (Achtman et al., 1999). In fact, their RNase E, PNPase, RhlB and enolase proteins are 97-100% identical. Unlike *Y. pestis* (which has caused three major human pandemics), *Y. pseudotuberculosis* (like the more distantly related *Y. enterotica*) causes a relatively benign self-limiting gastro-intestinal disease in humans (Galindo et al., 2011). Being psychrotropic and a human pathogen, a better understanding of *Y. pseudotuberculosis* stress responses could result in the discovery of novel targets for chemotherapeutic design. Both temperature (i.e. cold) and oxidative stress responses have been characterized in this manuscript, the former potentially experienced by *Y. pseudotuberculosis* or *Y. enterotica* during food processing and shipping and the latter experienced when attacked by host innate immune cells during an infection. Knowing that the exoribonuclease, PNPase, is required for cold growth of several organisms (Jones et al., 1987; Goverde et al., 1998) including *Y. pseudotuberculosis* (Rosenzweig et al., 2005), we strove to evaluate whether the PNPase requirement for cold growth of *Y. pseudotuberculosis* was degradosome-dependent. Similarly, we chose to characterize the *Y. pseudotuberculosis* oxidative stress response since PNPase had already been implicated in the *E.* H_2O_2 stress response in a degradosome-independent manner (Wu et al. 2009). In fact, PNPase has already been shown to promote yersiniae virulence and is required for optimal T3SS function (Rosenzweig et al., 2005; Rosenzweig et al, 2007), so

identifying the exact constituents of the *Y. pseudotuberculosis* degradosome improves our understanding of how RNA metabolism impacts bacterial virulence as well.

Our data have identified RhlB, PNPase, and RNase E as components of the *Y. pseudotuberculosis* degradosome which previously had been shown to only inlcude PNPase and RNase E (Yang et al., 2008). Furthermore, using the B2H assay, we demonstrated how the carboxy-terminus of a *Y. enterotica* derived RNase E protein can also interact with *Y. pseudotuberculosis* RhlB helicase strongly supporting the notion that all pathogenic yersiniae can assemble a degradosome. We further characterized the role the *Y. pseudotuberculosis* degradosome plays in various stress responses and surprisingly found that the *Y. pseudotuberculosis* degradosome is not implicated in all stress responses that require PNPase involvement. More specifically, we determined that the *Y. pseudotuberculosis* cold-growth requirement for PNPase (Rosenzweig et al., 2005; Rosenzweig et al., 2007) is degradosome independent. However, *Y. pseudotuberculosis* degradosome assembly was required for the oxidative stress response. Degradosome involvement with oxidative stress is in agreement with a previously published report of its requirement for macrophage-induced stress (Yang et al., 2008) and in contrast to its dispensability in the *E.* oxidative stress response (Wu et al. 2009). This is a shining example of how even closely related Gram-negative, enteric bacteria, e.g. *E.* and *Y. pseudotuberculosis*, might behave differently to various stressors and employ different coping mechanisms to overcome the stress itself.

Unexpectedly, PNPase and the degradosome affect growth during H_2O_2 stress in different phases of growth. PNPase appeared important during log-phase growth of *Y.*

pseudotuberculosis, while degradosome assembly affected biomass accumulation resulting in an early stationary phase. Even more unexpected was that the absence of PNPase suppressed the H_2O_2 sensitive phenotype of $RNE_{1\text{-}465}$. Furthermore, the deletion of the PNPase-encoding gene did not diminish expression levels of $RNE_{1\text{-}465}$, so the observation remains both intriguing and unexplained. In one scenario, PNPase responds to oxidative stress in *Y. pseudotuberculosis* independently during early growth; however, during later growth PNPase associates with the degradosome to overcome the stress and enter into an acclimation phase. Of course, such a scenario fails to explain the surprising and unexplained phenomenon in which the absence of PNPase suppressed the H_2O_2-sensitive phenotype of $RNE_{1\text{-}465}$. Perhaps a global evaluation of transcript abundance in each strain during oxidative stress is warranted and could reveal clues to help explain why PNPase and the degradosome affect growth during H_2O_2 stress differently despite PNPase not diminishing expression levels of $RNE_{1\text{-}465}$.

CHAPTER 5

CONCLUSIONS, FUTURE STUDIES, RECOMMENDATIONS

5.1 CONCLUSIONS

Taken together, these data have expanded our understanding of the *Y. pseudotuberculosis* degradosome by clearly identifying RhlB helicase as a member of the multi-protein complex. Additionally, these data have delineated the role of the *Y. pseudotuberculosis* degradosome in various stress responses. Whereas PNPase seemingly affects growth at 4°C in a degradosome-independent manner, the *Y. pseudotuberculosis* oxidative stress response clearly requires degradosome assembly to achieve optimal biomass during late log-phase growth. Realizing the unique contributions made by the degradosome during various stress responses could enable us to uncover novel chemotherapeutic targets more specifically aimed at disarming pathogens and making them more vulnerable/susceptible to those agents.

5.2 FUTURE STUDIES/RECOMMENDATIONS

Miccroarray studies have been conducted to provide a better interpretation of the mechanisms involved in YP (Darwin & Hortsman, 2012). However in this study we chose to use plate based studies in order to understand the functionally of the degradosome but one can surmise after understanding this work in fact there is a need to study these stress in the host cell of YPT. Specifically, conducting stress based studies in vivo of the Hela cell (Yang et al,). Being able to understand the mechanisms will help clinicians with treatment of Chron's disease and arthritis for example (Galindo et al, 2011). The pathogenic strains of yersiniae are significant regarding infection (Darwin &

Hortsman, 2012). Post-transcriptional studies and methodologies would provide a clearer mechanistic understanding of small regulatory RNAs such as csp transcripts, non-coding regions and more (Jones et al, 1987; Gripeland et al, 2010). Exploring in vivo techniques that encompases ini genes will show trends as opposed to solely looking at functionality (Darwin & Hortsman, 2012). Another option is to conduction a secretion assay and the a cell culture infection assay to determine what if any effector proteins could stabilize yersiniae that have been under abiotic stress.

APPENDICES

A. Keywords

- **Host Cell Immune Response**
- **Degradosome**
- **RNAase E**
- **PNPase**
- **Cold shock**
- **Oxidative Stress**

B. Abbreviations and Symbols

- *Yersinia pseudotuberculosis*.....YPT
- *Yersinia pestis*.....YP
- *Yersinia enterocolitica*.....YE
- °C.....degrees Celsius
- *Escheria coli*..... *E.coli*
- Ribonuclease.....RNAase
- *Polynucleotide Phosphorlase*..... PNPase
- RNA helicase B.....RhlB
- Cold shock proteins.....*csp*
- Cold inducible proteins.....*cip*
- Type Three Secretion System..... TTSS
- Reactive Oxygen Species.....ROS
- Superoxide anion.....(O_2^-)
- Hydrogen peroxide..... (H_2O_2)
- Hydroxyl radical..... (OH^-)
- Untranslated regions.....UTRs

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> 77004amandaj_henry@yahoo.com

Prof. Dr. Wolfgang Schumann
Institute of Genetics
University of Bayreuth
D-95440 Bayreuth – Germany
Phone: 0921 – 552708
Fax: 0921 – 552710
E-mail: wschumann@uni-bayreuth.de
http://www.genetik.uni-bayreuth.de/page/15/Research

Private address:
Donaustr. 8A
D-95445 Bayreuth
Phone: 0921-42794

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ABSTRACT

Bacteria require optimal growth conditions in order to thrive. However, stress factors such as cold and oxidation can attribute to the pathogenecity of a species. Ribonuclease E (RNase E) is a component of the multicomplex protein, the degradosome, which is inclusive of: enolase (a glycolytic enzyme), and RhlB helicase, and 3'-5' exoribonuclease polynucleotide phosphorylase (PNPase). The microbiological model organism, *E. coli*, is attributed to first indicating the presence of microdomain PNPase in the degradosome. However, the species of interest in this study is *Yersinia pseudotuberculosis* (YPT) due to its affinity to proliferate and sustain in said abiotic stress environments unlike its enterobacteriaceae counterpart. YPT exposed to oxidative stress need degradosome assembly therefore it is degradosome-dependent. Conversely, the cold growth mechanism of PNPase in YPT is degradosome-independent.